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(54) Title: GnRH ANALOGS FOR DESTROYING GONADOTROPHS

(57) Abstract

Certain toxic compounds (T) such as, for example, compounds based upon diphtheria toxin, ricin toxin, pseudomonas exotoxin, α-amanitin, pokeweed antiviral protein (PAP), ribosome inhibiting proteins, especially the ribosome inhibiting proteins of barley, wheat, corn, rye, gelonin and abrin, as well as certain cytotoxic chemicals such as, for example, melphalan and daunorubicin can be conjugated to certain analogs of gonadotropin-releasing hormone to form a class of compounds which, when injected into an animal, destroy the gonadotrophs of the animal's anterior pituitary gland. Hence such compounds may be used to sterilize such animals and/or to treat certain sex hormone related diseases.

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GnRH ANALOGS FOR DESTROYING GONADOTROPHS

FIELD OF THE INVENTION

The present invention generally relates to methods for sterilizing animals and to methods for medically treating certain sex hormone related diseases such as, for example, cancer of the breast or prostate. More particularly, this invention relates to sterilization and medical treatment by means of chemical attack upon the pituitary gland.

BACKGROUND OF THE INVENTION

Considerable interest exists with respect to the subject of sterilization of animals. This is especially true of those concerned with veterinary medicine and animal husbandry, particularly as they relate to the subject of sterilization of domestic animals such as dogs, cats, cattle, sheep, horses, pigs, and the like. Various methods have been developed over the years to accomplish sterilization. For example, with respect to male cattle, the most widely used procedure for eliminating problems of sexual or aggressive behavior is sterilization through surgical castration. This is done in various ways, e.g., crushing the spermatic cord, retaining the testes in the inguinal ring, or use of a rubber band, placed around the neck of the scrotum, to cause sloughing off of the scrotum and testes. However most

of these "m chanical" castration methods have proven to be undesirable in one resp ct or another; for example they (1) are traumatic, (2) introduce the danger of anesthesia, (3) are apt to produce infection, and (4) require trained personnel. Moreover, all such mechanical castration methods result in complete abolition of the testes and this of course implies complete removal of the anabolic effects of any steroids which are produced by the testes and which act as stimuli to growth and protein deposition.

These drawbacks have caused consideration of various alternative sterilization techniques such as the use of chemical sterilization agents. However, the use of chemical sterilization agents has its own set of advantages and disadvantages. On the positive side, chemical sterilization eliminates the stress and danger associated with mechanical castration. Chemical sterilization also has the added advantage of allowing for retention of certain anabolic effects resulting from a continued presence of low levels of circulating testosterone. This is especially valuable in the case of animals raised for human consumption since circulating testosterone promotes growth, efficiency of feed conversion and protein deposition. Unfortunately, there are several disadvantages associated with chemical sterilization. For example chemical sterilization is often temporary rather than permanent; it also sometimes produces extremely severe, and even fatal, side effects.

Many of these chemical sterilization methods have been aimed at regulation of luteinizing hormone produced at various stages of an animal's sexual development. For example, with respect to cattle it has been established that in the case of the infantile

calf, luteinizing hormone is rarely discharged and testicular production of androgens is at low levels. On the other hand, in a prepubertal calf, or an adult bull, discharges of luteinizing hormone from the anterior pituitary occur more frequently and the testes produce considerably larger amounts of testosterone and other steroids. It is thought that these conditions result from the following factors: (1) decreases in the concentration of estradiol receptors in the hypothalamus, (2) concomitant increases in the concentration of estradiol receptors in the anterior pituitary, and (3) increases the number of gonadotropin-releasing hormone (GnRH) receptors in the anterior pituitary. This increase in GnRH receptors is generally regarded as a prerequisite for an animal to pass from the infantile stage to the prepubertal and mature stages of endocrine development. Hence, based upon these understandings of the hypothalamic-pituitary-testicular axis, several chemical methods have been proposed to modify given animals, e.g., a bull calf, in such a way that it never enters puberty, but still receives stimuli for growth and protein deposition through the anabolic effects of steroids produced by modified testes. In any event, most of the chemicals proposed for such sterilization purposes are hormones or hormone analogs. For example U.S. Patent 4,444,759 teaches the use of a class of peptides analogous to GnRH (i.e., gonadotropin-releasing hormone, and particularly luteinizing hormone-releasing hormone) capable of inhibiting release of gonadotropins by the pituitary gland and thereby inhibiting release of the steroidal hormones, estradiol, progesterone and testosterone. It should also be noted that the terms "GnRH" (gonadotropin-releasing hormone) and "LHRH"

(luteinizing hormone-releasing hormone) ar sometimes used interchangably in the literature. For the purposes of describing the prior art both terms may be employed; however, for the purposes conveying the teachings of our patent disclosure, applicants prefer the term GnRH and will use it in describing their compounds.

Be that as it may, some prior art chemical sterilization procedures are specifically adopted to alter luteinizing hormone secretion before the animal has attained the age of puberty. This is not surprising since the role of luteinizing hormone in sexual maturation is well known. Luteinizing hormone is a gonadotropic hormone found in the anterior lobe of the pituitary gland and, in male animals, it is known to stimulate the interstitial cells of the testes to secrete testosterone (see generally, The Merck Index, 8th edition, p. 560 (1968), Encyclopedia of Chemical Technology, Vol. 7, pp. 487-488 (1951)).

One approach has been to use certain chemicals to produce antibodies in an animal which exhibit cross-reactivity with the gonadotropins produced by the animal's pituitary. It is generally thought that with such early antigenic stimulation, formation of antibodies is more continuously stimulated by the release of endogenous hormones and that early immunization with such luteinizing hormone deters the maturation of the gonads and adnexal glands. This, in turn, is thought to inhibit spermatogenesis at the spermatogonial level. However, early immunization of this kind also tends to make the interstitial tissues fibroblastic. It has also been found that such early stimulation of the immunologic system leads to development of a high titered antiserum to luteinizing hormone which remains at relatively high levels.

Nonetheless, periodic boosters are often necessary for adult animals sterilized befor puberty in order to maintain high levels of the n utralizing antibodies.

Similarly, luteinizing hormone has been administered to animals after they have attained the age of puberty in order to atrophy their reproductive organs and to cause a decrease in libido (see generally, M. Tallau and K.A. Laurence, Fertility and Sterility, Vol. 22, No. 2, February 1971, pp. 113-118, M.H. Pineda, D.C. Lueker, L.C. Faulkner and M.L. Hopwood, Proceedings of the Society for Experimental Biology and Medicine, Vol. 125, No. 3, July 1967, pp. 665-668, and S.K. Quadri, L.H. Harbers, and H.G. Spies, Proc. Soc. Exp. Biol. Med., Vol. 123, pp. 809-814 (1966). Such treatments also impair spermatogenesis in noncastrated adult male animals by interruption of the spermatogenic cycle.

Other chemical sterilization agents have been specifically designed for use on female animals. For example, it is well known that certain antigens will produce an antiserum against a requisite estrogen. This is accomplished by first making an antigen and then injecting said antigen into an animal for purposes of antiserum production. The animal is then bled to recover the antiserum. Any female animal of the same species as the host animal may then be injected with the antiserum at the proper time prior to ovulation and the injected antiserum will cause temporary sterilization of that animal.

Other methods of chemical sterilization have been based upon direct chemical attack upon certain cells of the pituitary itself (as opposed to chemical attacks upon the hormone products of such cells) with a view toward permanently destroying such cells. Again, this approach is suggested by the fact that

follicle stimulating hormon (FSH) and luteinizing hormone (LH) (sometimes r ferred to as gonadotropins or gonadotropic hormones) are released by the pituitary gland to regulate functioning of the gonads to produce testosterone in the testes and progesterone and estrogen in the ovaries. They also regulate the production and maturation of gametes.

Several chemical agents have been proposed for this purpose. However, it has been found that most chemical agents which are in fact capable of destroying the gonadotrophs of an animal's anterior pituitary gland also tend to produce extremely toxic side effects which can severely weaken, and sometimes kill, the treated animal. Hence, with respect to the general subject of chemical sterilization, it can be said that any chemical capable of producing sterilization without, or with minimal, toxic side effects would be of great value in the fields of animal husbandry, veterinary medicine and wildlife control.

Knowledge of the above noted sex hormone functions also has produced several advances in the field of human medicine. For example, the potential for achieving chemical castration (rather than "surgical" castration) with certain luteinizing hormone-releasing hormone (LHRH) analogs has been reported (see for example, Javadpour, N., Luteinizing Hormone-Releasing Hormone (LHRH) in Disseminated Prostatic Cancer; 1M, Vol. 9, No. 11, November 1988). Table I below gives the structure of LHRH and the structure of certain analogs (e.g., Goserelin, Leuprolide, Buserelin and Nafarelin) of LHRH which are capable of temporarily suppressing luteinizing hormone secretion and thereby suppressing the gonads. As a consequence, these LHRH analogs have come to be regarded as a promising new

class of agents for the treatment of various host-dependent diseases, especially prostatic cancer. In referring to Table I, it first should be noted that LHRH has a decapeptide structure and that substitution of certain amino acids in the sixth and tenth positions of the LHRH produce analogs which render agonists that are up to 100 times more potent than the parent LHRH compound (hence these compounds are often referred to as "superagonists"). Structures of LHRH and the most commonly known LHRH superagonists are listed in Table I.

TABLE I

STRUCTURES OF LHRH AND SOME SUPERAGONISTS
(Superagonists have substitutions at positions 6 and 10)

LHRH: pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-Amide

pGlu	His	Trp	Ser	Tyr	Gly	Leu	Arg	Pro	Gly-Amide
1	2	3	4	5	6	7	8	9	10

SUPERAGONISTS:

Name	Subs. at 6	Subs. at 10	Terminator
Goserelin:	D-Ser(tBu)	AzaGly	Amide
Leuprolide:	D-Leu	des-Gly	Ethylamide
Buserelin:	D-Ser(tBu)	des-Gly	Ethylamide
Nafarelin:	D-2-NaphthylAla	None	Amide

While these compounds represent the most promising means for palliative therapy because of their relative lack of side effects, they remain expensive and must be administered repeatedly. Even the newest formulations utilizing polymer encapsulated drug or other depot forms will require at least monthly administration.

Depot forms are presently in development, but they

too are likely to be qually expensive and also requir monthly administration. In response t these drawbacks, Applicants have develop d a class of compounds which is capable of producing safe, inexpensive, chemical castration as an alternative to surgical castration. Such drugs also greatly simplify therapy of the generally elderly patients with prostate cancer, and could eliminate the need for surgical castration (still preferred by many urologists) as well as provide a medical alternative to oophorectomy in females with advanced breast cancer. Moreover, as a model system, the ability to eliminate pituitary gonadotrophs in vivo, which are regulated by GnRH receptors in response to ligand stimulation in a predictable fashion, is a highly appealing first step toward the more complex use of toxin conjugates to antibodies to eliminate tumor targets. Hence, use of applicants' compounds generally will fall into two major areas of use. The first is sterilization of mammals of all types; the second is chemical castration of mammals in general, and human beings in particular, for purposes of treating breast or prostate cancer by ablating those pituitary cells, namely gonadotrophs responsible for LH secretion.

SUMMARY OF THE INVENTION

The present invention provides a group f toxin compounds, agents and processes for effectively sterilizing animals (e.g., mammals such as cattle, sheep and humans, as well as fowl and fish) and/or for treating certain sex hormone related diseases such as cancer of the prostate and cancer of the breast, especially in humans. These compounds also have utility as agents for aiding in putting weight on (e.g., increasing the rate of metabolic formation of protein) cattle and other meat producing animals. These compounds or agents may also be referred to as "toxic compounds", ("T") or "toxins" for the purposes of this patent disclosure. In any event, the most effective, and hence most preferred of these compounds (toxins) are based upon diphtheria toxin, ricin toxin, pseudomonas exotoxin, shiga toxin, α-amanitin, pokeweed antiviral protein (PAP), ribosome inhibiting proteins (RIP), especially the ribosome inhibiting proteins of barley, wheat, flax, corn, rye, gelonin, abrin, modeccin and certain cytotoxic chemicals such as, for example, melphalan, methotrexate, nitrogen mustard, doxorubicin and daunorubicin. All of these toxins are characterized by their lack of ability, in their own right, to chemically attack the gonadotropin-secreting cells of the anterior pituitary gland.

Applicants have, however, found that these sterilization agents can be effectively delivered to the pituitary gland while chemically conjugated with certain peptide hormone molecules such as certain analogs of gonadotropin-releasing hormone, GnRH. Other peptide hormone molecules (other than gonadotropin-releasing hormone) to which the herein disclosed toxins could be conjugated are human

chorionic gonadotropin, equine chorionic gonadotropin, luteinizing hormone and follicle-stimulating hormone. At this point, it should also be noted that for the purposes of this patent disclosure, the term gonadotropin-releasing hormone will usually be abbreviated as "GnRH". However, it should also be noted that for the most part certain hereinafter described analogs of GnRH are far more effective carrier peptide hormone molecules for the practice of this invention than the basic or parent GnRH molecule. In their most generalized sense, these analogs will be abbreviated as "GnRH-A", with the "A" designating that the resulting compound is an analog of the basic GnRH molecule. Similarly, in their most generalized sense, any general toxin compound which is conjugated to the GnRH-A molecule will be abbreviated by the letter "T" for toxin. Thus the abbreviation for a generalized conjugate of the GnRH-A and toxin will be "GnRH-A-T".

In certain hereinafter described preferred cases the concept of an "analog" of the GnRH also will include those molecules wherein the analog includes a linkage or coupling moiety specifically intended to link the GnRH-A group or moiety to the toxic group or moiety. Most preferably such toxic compounds (T) and their associated carrier peptide molecules are covalently linked by a linking or coupling agent selected from the group consisting of N-succinimidyl 3-(2 pyridyldithio) propionate (SPDP), 2-iminothiolane, thioether, carbodiimide, and epsilon amino caproic acid. In the case of a GnRH-A carrier peptide molecule, this linking or coupling is preferably carried out at the 6 position of the parent GnRH molecule. That is to say that most preferable production of such "analogs" of the basic GnRH molecule will involve modification of the 6 position

of the GnRH molecule. Amino acid substitutions at th 6 position of the GnRH molecule yield analogs with high affinity for GnRH receptors and also provide a means for covalently linking the toxin molecule to the GnRH analog. The most preferred amino acids for this purpose are lysine, D-lysine, aspartic acid, D-aspartic acid, glutamic acid, D-glutamic acid, cysteine, D-cysteine, ornithine, D-ornithine tyrosine, D-tyrosine, and other amino acids having suitable side-chain functional groups such as, for example, amino groups, carboxylic groups or sulfhydryl groups. Similarly the 10 position of the basic GnRH molecule can be modified to produce other analog variations. Addition of groups such as Gly-NH_2, ethylamide, or Aza-Gly-NH_2 to the Pro at the 9 position of the basic GnRH molecule produce preferred analog forms. A structural depiction of the most preferred members of the resulting group of compounds of this patent disclosure is given in TABLE II.

TABLE II

(Preferred GnRH-A-T Compounds)

```
pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Z
  1   2   3   4   5   6   7   8   9  10
                      |
                      X
                      |
                      Y
                      |
                      T
```

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Wherein the pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro
              1   2   3   4   5   6   7   8   9-Gly
```

10 represents the GnRH group, X is an amino acid selected from the group consisting of: tyrosine, D-tyrosine, ornithine, D-ornithine, glutamic acid, D-glutamic acid, aspartic acid, D-aspartic acid,

cysteine and D-cysteine; Y is a linking agent (wh se use is optional, but highly preferred) selected from the group consisting of: carbodiimide, 2-iminothiolane, SPDP, (N-succinimidyl 3-(2-pyridyldithio) propionate), bis-diazobenzidine and glutaraldehyde; Z is a chemical group selected from the group consisting of Gly-NH_2, ethylamide, and Aza-Gly-NH_2 and T is a toxin group, capable of chemically attacking the gonadotrophs of the pituitary gland when conjugated to the carrier peptide molecules described in this patent disclosure. Such toxins can be selected from the group consisting of the plant toxins: ricin, modeccin, abrin, pokeweed anti-viral protein, α-amanitin, gelonin ribosome inhibiting protein ("RIP"), barley RIP, wheat RIP, corn RIP, rye RIP and flax RIP; the bacterial toxins: diptheria toxin, pseudomonas exotoxin and shiga toxin and chemical toxins: melphalan, methotrexate, nitrogen mustard, doxorubicin and daunomycin.

Again, the most preferred forms of these compounds will have the toxin T group (or T groups) attached to the linkage group Y at the site of an amino group, a carboxylic group or a sulfhydryl group of the Y group. In other words the T group will be attached at an amino, carboxylic or sulphydral group of the carbodiimide, the 2-iminothiolane, SPDP, bis-diazobenzidine or the glutaraldehyde which is used as the linkage group Y. Similarly, the Y group will be attached to the X group at the site of an amino group, a carboxylic group or a sulphydral group of the amino acid group which is employed as the X group of the overall compound.

Some of the more preferred compounds made according to this patent disclosure are based upon the following additional, preference considerations

regarding compounds comprised of a hormone group, a linking agent group and a toxin group. The most pref rred hormones that may b used to produce our hormonotoxins for chemical castration would include: (1) (pyro)Glu-His-Trp-Ser-Tyr-D-Lys-Leu-Arg-Pro-Ethylamide D-Lys^5-GnRH-Pro^9-Ethylamide, (2) luteinizing hormone (LH) or (3) interstital-cell stimulating hormone (ICSH). Similarly, the most useful linking agents to produce hormonotoxins would include: (1) Succinimidyl 4-(p-maleimidophenyl) butyrate (SPDP) and (2) N-Succinimidyl 3-(2-pyridyl-dithio) proprionate (SMPB). Finally, the most promising toxins for production of hormonotoxins would include (1) Diphtheria toxin A chain and (2) Pseudomonas exotoxin A chain. Within the possible compounds which could be made by combining the above hormones, linking agents and toxins, two compounds are especially preferred. They are: (1) D-Lys^6-GnRH-SMPB-diphtheria toxin A chain conjugate and (2) D-Lys^6-GnRH-SMPB-speudomonas exotoxin A chain conjugate. It should also be noted in passing that the toxins can either be derived from natural sources or produced using recombinant DNA technology.

Applicants have found that GnRH conjugations of the types noted in TABLE II above are particularly effective in causing the toxic compound T to be specifically targeted to the gonadotropin-secreting cells of the anterior pituitary gland. Indeed they are the only cells to which the gonadotropin-releasing hormone portion of the conjugate will bind. Hence, these toxic compounds, bound to an analog of gonadotropin-releasing hormone, can be employed to permanently destroy a subpopulation of the anterior pituitary cells and thereby eliminate the gland's ability to secrete gonadotropins. This in turn causes

the animal's gonads t atrophy and lose their ability to function for reproductive purposes. That is to say that, without functioning gonadotrophs, an animal is not able to secrete luteinizing hormone (LH) and follicle-stimulating hormone (FSH) and thus is rendered sterile. Applicants have postulated that the compounds of this patent disclosure inhibit synthesis of LH, and presumably other proteins made by gonadotrophs, because they tend to inhibit all protein synthesis once these compounds gain entry into a cell. It should also again be noted that applicants' compounds act as GnRH superagonist. Hence, use of applicants' compounds allows "chemical castration" to be employed in place of surgical castration.

Thus, the use of these compounds has great utility in human medicine as well as in veterinary medicine. This follows from the fact that there are several important biological reasons for employing castration and antifertility drugs in humans. For example, breast and prostate cancers are but two examples of sex steroid-dependent tumors which respond to such hormonal manipulation. At present, the only reliable way to inhibit steroid-dependent tumor growth is through administration of counter-regulatory hormones (e.g., DES in prostate cancer), sex-steroid hormone binding inhibitors (e.g., tamoxifen in breast cancer) or surgical castration. Thus the potential medical uses of such chemical castration compounds are vast and varied. For example, prostate cancer remains an important cause of cancer deaths and represents the second leading cancer of males. The present palliative treatment for advanced prostate cancer cases involves reduction of serum testosterone/DHT levels through use of surgical castration. It should also be noted that for purposes of disease and/or

fertility control, especially in humans, it may be desirable to use applicants' compounds to ablate pituitary gonadotr phs in conjunction with other modes of treatment. For example, it is anticipated that chronic administration of progestins and estrogens to females and androgens to males might be necessary to prevent loss of secondary sex characteristics, behavior and osteoporosis. However, through judicious use of the herein disclosed compounds, especially in combination with appropriately administered sex steroids, desirable antifertility effects can be achieved. Another area of application in human medicine is treatment of endometriosis. This condition, which produces painful growth of endometrial tissue in the female peritoneum and pelvis also responds to inhibition of sex steroid synthesis. Those skilled in this art will also appreciate that the herein disclosed compounds could be used to partially reduce sex-steroid secretions, and thus reduce or eliminate certain hormone related behavior problems while retaining improved growth stimulation.

The dose/time adjustments associated with the use of these compounds can vary considerably; however, these compounds are preferably administered by injection into a mammal in concentrations of from about 0.1 to about 10 milligrams per kilogram of the mammal's body weight. Sterilization may be accomplished with as few as one injection; but multiple treatments (e.g., based upon concentrations of from about 0.03 milligrams once every 4 days to about 1 milligram per kilogram of body weight for 20 days) are alternative sterilization schemes. Furthermore, as sterilization agents, the compounds of this patent disclosure can be used before or after puberty. They too are especially useful in those

areas f animal husbandry wher th anabolic benefits of non-surgical sterilization techniques can contribute to meat production and/or quality. In one preferred embodiment of this invention the compounds of this invention are administered to male cattle between the ages of about 8 weeks and 20 weeks at least once and in a concentration of from about 0.1 to about 10 milligrams per kilogram of the animal's body weight.

The toxic moieties T of the herein disclosed compounds are obtainable from both natural and synthetic sources. For example, pokeweed antiviral protein can be isolated from leaves of pokeweed plants and purified by gel filtration chromatography. It can then be, by way of example, conjugated to D-Lys^6-$desGly^{10}$-pro^9GnRH-ethylamide via the amino group on the lysine and through a sulfhydryl group introduced into the pokeweed antiviral protein by a heterobifunctional reagent. In any event, one of the chief advantages of these compounds is their ability to produce permanent sterilization without strong toxic side effects. Hence these compounds may be used on mammals such as human beings, domestic animals, pets or wild animals. Moreover, they can be administered as a single injection which can induce permanent and irreversible sterility in both male and female mammals. However, an alternative approach to achieve sterilization is through multiple injections at lower dosages than those employed in a single treatment or by slow release implants (i.e., biodegradable formulations).

Applicants also have postulated that the "B-chain" portion of their toxic moieties are important not only for binding to cell surfaces, but for trans-membrane translocation of their A-chain. This was particularly demonstrated for the A-chain of Diphtheria toxin,

Ricin and Pseudomonas exotoxin. To this end, applicants prepared conjugates of GnRH-A to A and B chains of Diphtheria t xin as well as to a modified A-B chain which was genetically engineered to eliminate the carboxy terminal binding portion of the B-chain. These conjugates were shown to bind to pituitary cell GnRH receptors. They were found to possess enhanced toxicity over A-chain conjugates based on improved trans-membrane transport characteristics. Given this, those skilled in the art will appreciate that numerous genetic and chemical modifications of B-chains should allow further exploitation of this approach. That is to say that, by such methods, it is possible to generate a whole series of conjugates that can be characterized as GnRH-A-A/B, GnRH-A-A, GnRH-A-A plus GnRH-B, all of which could enhance the findings described herein by simultaneous delivery of membrane active B-chains with the herein described GnRH-A-T conjugates.

DESCRIPTION OF DRAWINGS

Figures 1A and 1B respectively depict the results of GnRH induced secretion of LH based upon a singl injection of a GnRH-A-T compound and the results of GnRH induced secretion of LH based upon 4 injections of a GnRH-A-T compound.

Figure 2 indicates inactivation of certain grain hemitoxins (wheat hemitoxin and barley hemitoxin) by SPDP conjugation.

Figure 3 depicts the results of a SDS-PAGE analysis of carbodiimide conjugated hemitoxins.

Figure 4 shows the inhibition of 2-iminothiolane-conjugated barley hemotoxin.

Figure 4A shows SDS-PAGE analysis of barley hemitoxin after conjugation to [D-Lys^6, des-Gly^{10}]-GnRH-ethylamide using 2-iminothiolane.

Figure 5 shows binding curves indicating the ability of [D-Lys^6, des-Gly^{10}]-GnRH-ethylamide toxin conjugates to bind to pituitary receptors.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

One of the chief objects of this invention is to provide a class of compounds which will allow safe, inexpensive, chemical castration. As such, applicants' compounds represent an alternative to surgical castration as well as to surgery for treatment of diseases such as breast cancer or certain sex hormone related prostate cancer. In order to better define this class of compounds, Applicants conducted studies on various linking technologies as they apply to numerous toxin candidates. These studies resulted in the herein disclosed group of conjugate compounds. In general these compounds display exceptional gonadotroph membrane binding characteristics along with full retention of toxin

activity.

In general, the sterilization activity of the compounds of this patent disclosure was tested in receptor binding assays (to be sure a given conjugate was still capable of interacting with the GnRH receptor cells of the pituitary), in a cell-free translation system (to insure that the toxic protein maintained its toxicity), in cell culture systems (to determine if a given toxic conjugate is capable of inhibiting synthesis of LH), and in test animals (to determine if sterility was induced). The most effective of these sterilization agents found to this point in Applicants' research is D-Lys^6-desGly^{10}-pro^9-GnRH-ethylamide conjugated to pokeweed antiviral protein using carbodiimide as the linkage between the carrier protein molecule and the toxin moiety.

Again, a distinct advantage of each of the sterilization agents of this invention, and pokeweed antiviral protein in particular, is that they have an extremely limited ability to enter cells in an animal's body unless they are first conjugated to a carrier such as gonadotropin-releasing hormone. Such conjugation can be accomplished in several ways. By way of example, pokeweed antiviral protein can be conjugated to a [D-Lys^6, des-Gly^{10}]-GnRH-ethylamide molecule via the ϵ-amino group on the Lysine and through a sulfhydryl group on the pokeweed antiviral protein.

By way of example, applicants found that this type of linkage reduces the ability of the [D-Lys^6, des-Gly^{10}]-GnRH-ethylamide to bind to the GnRH receptor by 99%. In addition, the conjugation procedure reduces the toxicity of the pokeweed antiviral protein by 99.5 in a cell-free translation system. However, despite large reductions in activity of both the GnRH analog

and the st rilization agent by this particular conjugation procedure, some activity of each was maintained. The activity of this conjugate was also tested in a pituitary cell culture system. In this system, pituitary cells were incubated with the sterilization agent conjugated to [D-Lys^6, des-Gly^{10}]-GnRH-ethylamide for 16 hours. After incubation, the sterilization agents were removed from the incubation media by extensive washing and the cells were then cultured for an additional 24 hours. The increase in total LH, i.e., that present in the media plus that in the cells during the 24 hour period, represents the ability of the treated cells to synthesize LH. Using this system, it was established that these toxic conjugates can completely inhibit synthesis of LH by the cultured cells. Compared to control cells, the sterilization agent alone reduced synthesis of LH by only 20%. Thus, by this method, it was established that the compounds of this patent disclosure can inhibit synthesis of LH and presumably other proteins made by gonadotrophs since this class of compounds has the ability to inhibit all protein synthesis once they gain entry into a cell.

Applicants also tested these compounds using an in vivo model. The test system initially chosen was the ovariectomized female rat. The parameter examined was GnRH induced secretion of LH. The results of such an experiment with rats are shown in Fig. 1A. It indicates that a single injection of a toxic conjugate (i.e., GnRH-A-T) wherein the toxic moiety (T) pokeweed antiviral protein and the GnRH-A moiety was [D-Lys^6, des-Gly^{10}]-GnRH-ethylamide. During week 1, this compound induced secretion of LH equivalent to that of GnRH-A alone. This indicated that the sterilization agent conjugate was binding to the GnRH receptor in

vivo. During week 2, release of LH was reduced by 50% in the GnRH-A treat d group (controls), but by >90% in the GnRH-A-T group. By the third week, the release of LH in the GnRH-A-T group had returned to the same level as that observed in the control animals. This indicated that a single treatment with the sterilization agent conjugate was probably not sufficient to completely kill the gonadotrophs in vivo. It might however be the basis for a temporary sterilization. Based upon this finding, a second experiment was conducted to examine the effect of 4 injections of a pokeweed antiviral sterilization conjugate at 3-day intervals on the ability of ovariectomized rats to release LH. In this experiment, the rats were unable to release LH in response to GnRH stimulation one month after initiation of the treatment (Fig. 1B). These data strongly indicate the ability of these conjugates to permanently inhibit reproduction in intact male and female animals.

In another set of experiments, intact rats were given 4 injections of GnRH-A-T compounds, again wherein the toxic moiety T was selected from pokeweed antiviral protein, ricin A chain, and ribosome inhibiting proteins, of certain grains (again, those of wheat, corn, barley and rye,) at 3-day intervals and their subsequent reproductive capacity was compared to rats treated with only the respective toxin T or to that of untreated rats. In this experiment, treatment of male rats with only the toxin T did not reduce their fertility compared to controls (percentage of females that became pregnant was 100%). However, fertility was greatly reduced in those males that were treated with a GnRH-A-T agent such as, for example D-Lys^{6}-desGly^{10}-pro^{9}-GnRH-ethylamide con-

jugated to pokeweed antiviral protein, i.e., only 50% of the females exposed to males became pregnant. Moreover, fertility did not appear to increase with time after treatment. Histological examination of the testes of these rats indicated that most of the seminiferous tubules were devoid of sperm. However, 10% of the tubules appeared to still be producing sperm and probably accounted for the pregnancies observed. The weight of the testes was reduced by nearly 50% and did not recover within 6 months after the end of treatment. Thus, the effects of the treatment appeared to be permanent and dose related. Female rats treated with the toxic conjugate were sterile and remained so for at least 4 months after the end of treatment. Most important is the fact that none of the rats treated with the toxic conjugate appeared to have any side effects.

EXEMPLARY CHEMICAL EXPERIMENTAL METHODS

1. Synthesis of [D-Lys^6, des-Gly^{10}]-GnRH-ethylamide. Synthesis of this analogue was accomplished using the solid phase method on hydroxymethyl resin and cleavage from the resin by ethyl amine, yielding the ethylamide. Following HF cleavage of protecting groups from side chains the peptide was purified by countercurrent distribution, purity of the peptide was assured by TLC, paper electrophoresis, and amino acid analysis of the acid hydrolysate.

2. Applicants also produced a caproic acid derivative (134.91 mg) and the lysosomal hydrolase sensitive tetrapeptide spacer Leu-Ala-Leu-Ala-D Lys^6 (16.25 mg).

3. Conjugation of [D-Lys^6, des-Gly^{10}]-GnRH-ethylamide to toxins using SPDP. Applicants

endeavored to construct toxic conjugates of [D-Lys^6, des-Gly^{10}]-GnRH-ethylamide with the ricin A-chain. At the time these studies were initiated, Ricin A-chain was commercially available, but applicants found it to be both expensive and very unstable to temperature changes or conjugation procedures. Construction of an effective hemitoxin [D-Lys^6, des-Gly^{10}]-GnRH-ethylamide conjugate requires coupling of hemitoxin to hormone via a protein cross-linking reagent that does not block either the enzymatic activity of the hemitoxin or the binding specificity of the hormone. Therefore, applicants investigated a number of different hemitoxins in addition to ricin A and pokeweed antiviral protein and a number of different conjugation techniques. This work was largely directed at purification of certain plant hemitoxins, i.e., ribosomal inhibitory proteins, ("RIP"), a relatively recently recognized group of proteins which share the ability to enzymically inactivate mammalian ribosomes. Such toxins are potentially promising as alternatives to the more familiar A-chains of, for example, ricin in that they do not require separation from the cell-binding B-chains. The bi-functional coupling reagent most commonly used for this purpose is N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP). This compound forms covalent linkages to either free amino or sulfhydryl groups on proteins, but SPDP normally is attached to amino groups in hemitoxins, partly because many hemitoxins do not contain sylfhydryls that are available for coupling.

Initial experiments examined the reaction of SPDP with both the wheat and barley hemitoxins at various SPDP: hemitoxin ratios. The reactions were carried out at pH 9 for 30 minutes at 23°C at a protein concentration of 0.6 mg/ml. After 30 minutes a 20-

fold molar excess (over SPDP) of lysine was added to react with free SPDP and the hemitoxins diluted and assayed for inhibition of polyphenylalanine synthesis on Ehrlich ascites cell ribosomes. The results are presented in Figure 2.

Figure 2 is intended to show inactivation of certain grain hemitoxins by SPDP conjugation. It indicates that even 1:1 ratios of SPDP to hemotoxin result in significant inactivation which is complete at a 20:1 ratio. A commonly used 2-3 fold ratio would result in >95% inactivation. Applicants' study was expanded to include hemitoxins from corn and pokeweed. Reactions were carried out in phosphate buffers at neutral and acidic pH's in anticipation that under acidic conditions differences in pKa of lysine amino groups or conformational changes in some of the proteins might protect enzymic activity. However, in all conditions and with all 4 hemitoxin proteins, significant inactivation occurred and as quantitative activity measurements of hemitoxins were rather imprecise; hence applicants were unable to conclude that residual activity was not from unreacted hemitoxin. Moreover, these particular experiments indicated SPDP would be unsuitable as a coupling reagent for preparing many GnRH-A-T conjugates.

4. Conjugation of [D-Lys^6, des-Gly^{10}]-GnRH-ethylamide to toxins using Carbodiimide. Applicants examined the ability of the water soluble coupling reagent, carbodiimide linkages in this class of compounds. Although carbodiimide has been used successfully for coupling polypeptide hormones to proteins, applicants are unaware of any studies reporting its use in preparing toxin-protein conjugates. However, its use turned out to be attractive since it couples through carboxyl groups on

the hemitoxin rather than amino groups. It should also be noted that applicants' synthetic GnRH analogs are blocked at the carboxyl and amino termini, thus leaving, for example, D-lys^6 amine as the only reactive moiety. Use of large molar ratios of GnRH favors reaction of the hemitoxin to the analog rather than to itself.

Figure 3 shows the successful results of this approach. It represents a SDS-PAGE analysis of carbodiimide conjugated hemitoxins. In order to carry out these experiments, a 30:1 molar ratio of D-Lys^6, des-Gly^{10}] - GnRH - ethylamide to hemitoxin was reacted with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) in water at 23°C for 30 minutes and the reaction mixture passed through a Bio-Gel P6 column to desalt the product. Protein containing fractions were assayed for residual activity (see text) and the reaction products examined by SDS polyacrylamide gel electrophoresis. Lanes 1,and 6 are standards; lane 2, barley; lane 3, barley-GnRH; Lane 4, pokeweed; lane 5, pokeweed-GnRH; lane 7, rye-GnRH; lane 8, rye; lane 9, gelonin-GnRH; lane 10, gelonin. Conjugation in each case resulted in a 32kDa product which was distinct from the 30 kDa hemitoxin alone, and which (by enzyme assay) retained 10% of the original activity. Hemitoxins from barley, rye, wheat and the unrelated pokeweed and gelonin hemitoxins have each been successfully conjugated in this fashion and all retain about 10% of original toxicity in ascites ribosomal assay. Biologic studies with these conjugates were then completed in the manner hereinafter described.

5. Conjugation of [D-Lys^6, des-Gly^{10}]-GnRH-ethylamide to toxins using 2-iminothiolane. Although 2-iminothiolane, like SPDP, reacts with free amino

groups on proteins, it does not affect the activity of gelonin or PAP. Applicants have hypothesized that perhaps the reason 2-iminothiolane differs from SPDP in this regard is that it reacts with a different amino group on the protein or that it places a positive charge on the active amino group and thereby preserves enzymatic activity. In any case, applicants reacted 2-iminothiolane with barley hemotoxin at several reagent: protein ratios, separated the protein from unreacted 2-iminothiolane by gel exclusion chromatography on Sephadex G-25 and quantitated the amount of sulfhydryl groups introduced onto the hemitoxin by sulfhydryl exchange with the reactive, chromogenic disulfide 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB). The derivatized barley hemitoxin preparations were assayed for their ability to inhibit protein synthesis in ascites cell-free extracts and were found to have retained full activity.

Figure 4 depicts inhibition of protein synthesis by 2-iminothiolane-conjugated barley hemitoxin. Barley hemitoxin was incubated at 0°C for 90 minutes with 0 (o), 8-fold (x) or 24-fold (o) molar excess of 2-iminothiolane. The derivatized hemitoxins were then assayed for their ability to inhibit protein synthesis in ascites cell-free extracts. Proteins contained 0 (o), 0.76 (x) and 1.44 (o) moles of 2-iminothiolane bound per mole of hemitoxin.

Conjugation between the barley hemitoxin and [D-Lys^6, des-Gly^{10}]-GnRH-ethylamide was carried out by disulfide exchange. A sulfhydryl group was introduced into [D-Lys^6, des-Gly^{10}]-GnRH-ethylamide by reacting the hormone with a 16- fold molar excess of 2-iminothiolane at 0°C for 2 hours. Derivatized [D-Lys^6, des-Gly^{10}]-GnRH-ethylamide was separated from unreacted 2-iminothiolane by chromatography on a Bio-

Gel P-2 column equilibrated with 30% acetic acid. Acetic acid was removed from the isolated hormone by rotary evaporation followed by lyophilization. A reactive disulfide was prepared from barley hemitoxin as described above by incubating the hemitoxin with a 24-fold molar excess of 2-iminothiolane, isolating the protein and reacting it with DTNB to prepare the disulfide, and separating the hemitoxin from unreacted DTNB by column chromatography on Sephadex G-25. A 12-fold molar excess of derivatized [D-Lys^6, des-Gly^{10}]-GnRH-ethylamide was added to hemitoxin disulfide and disulfide exchange permitted to occur overnight at 4°C. Hemitoxin was separated from unconjugated GnRH by Sephadex G-25 column chromatography.

The reaction products were analyzed by SDS-polyacrylamide gel electrophoresis under non-reducing conditions. Analysis showed that the coupling reaction had converted approximately 50% of the 29 kDa barley hemitoxin (track 5) into a 31 kDa product (tracks 1-4) corresponding to a 1:1 hemitoxin- [D-Lys^6, des-Gly^{10}]-GnRH-ethylamide conjugate. The faint band of unreacted [D-Lys^6, des-Gly^{10}]-GnRH-ethylamide that can be seen in track 1 migrating ahead of the 14 kDa marker disappeared following acetone precipitation of the hemitoxin (track 2) or gel exclusion chromatography on Sephadex G-25 (tracks 3 & 4). The mixture of conjugate and unreacted hemitoxin was not purified further but was assayed directly for pituitary cell binding and killing.

Figure 4A depicts SDS-PAGE analysis of barley hemitoxin after conjugation to [D-Lys^6, des-Gly^{10}]-GnRH -ethylamide using 2-iminothiolane. Reaction products were analyzed before (tracks 1 & 2) and after tracks 3 & 4) Sephadex G-25 chromatography, and before (tracks 1 & 3) and after (tracks 2 & 4) concentrating

by acetone precipitation. Track 5 contained unreact d hemitoxin.

6. Conjugate Binding Studies. In order to assess whether [D-Lys^6, des-Gly^{10}]-GnRH-ethylamide toxin conjugates retain their ability to bind to receptors, the following assay was devised. Various concentrations of each conjugate were evaluated for their ability to displace 50,000 cpm ^{125}I-D Ala^6-GnRH from bovine pituitary membranes. After incubation for 4 hours in standard conditions at 4°C, membranes were pelleted, counted in a gamma counter to determine the bound labelled ligand, and the ability of each conjugate to displace 50% of the label (IC$_{50}$ for unlabelled [D-Lys^6, des-Gly^{10}]-GnRH-ethylamide. Figure 5 indicates the results of binding curves obtained in these experiments. Also shown are the calculated number of molecules required to displace 1 molecule of unconjugated [D-Lys^6, des-Gly^{10}]-GnRH-ethylamide. For example, Figure 5 shows competitive binding of toxin conjugates to bovine pituitary membranes. The abbreviations are: 21T, 2-iminothiolane; PAP, Pokeweed Antiviral Protein; SPDP, N-succinimidyl 3-(2-pyridyldithio) propionate; CI, Carbodiimide; EACA, Epsilon-amino caproic acid linker. Grain names refer to the purified hemotoxin source.

The data in Figure 5 was critical in determining applicants' next steps. Several conclusions were reached. First, SPDP severely limits toxin activity (see Figure 2). It also produces conjugates with greatly reduced binding activity (compare PAP-SPDP with Barley carbodiimide). On the other hand, use of carbodiimide produced conjugates with 3-40 fold improved binding compared to SPDP. However, there were differences among the hemitoxins used. For example, the wheat, rye and gelonin carbodiimide

conjugates all showed greater binding than did the barley carbodiimide conjugate. However, the barley carbodiimide conjugate retained greater toxicity than the other grain hemitoxin conjugates in the cell free protein synthesis assay (data not shown). In this case, use of a spacer arm actually decreased binding affinity. Finally, the 2-iminothiolane conjugate made with barley hemitoxin as described above retained both 100% toxicity in the cell free system (see generally Figure 4) and was as active as the best of the carbodiimide conjugates in binding. Applicants noted a 4.5 fold reduction in binding compared to the unconjugated [D-Lys^6, desGly^{10}]-GnRH-ethylamide. This was quite acceptable since native GnRH has also only about 1/30 the binding activity as this analogue (data not shown). Thus, after this exploratory work was completed, applicants carried out most further work with either the PAP-SPDP-[D-Lys^6, desGly^{10}]-GnRH-ethylamide or the barley 2-imminothiolane [D-Lys^6, desGly^{10}]-GnRH-ethylamide conjugate.

In Vitro Experiments.

The effect of these compounds on ovine pituitary cells in suspension culture was measured. A pituitary was removed from a ewe, sliced thinly, and dissociated with a mix of collagenase, hyaluronidase, and DNAase. The cells were washed several times and resuspended in culture medium containing 30% ram's serum. Cells were cultured in a 37° shaking water bath in 50 ml flasks under 95% O_2/5% CO_2. In a typical experiment, cells were divided into four groups after dissociation and cultured overnight (20 hr) with 1) culture medium only, 2) 10^{-8} M GnRH, 3) 3×10^{-9}M Toxin-[D-Lys^6, desGly^{10}]-GnRH-ethylamide (molarity expressed in terms of GnRH receptor binding activity) and 4) Toxin at the same concentration as Toxin-[D-Lys^6, desGly^{10}]-GnRH-

ethylamide. After pretreatm nt, the cells were washed 6 times, counted, and small aliquots removed for testing. The remainder were cultured in plain m dium for 24 hours. To test the cells, aliquots of 500,000 cells were washed and resuspended in challenge medium containing 10^{-7}M GnRH for 2 hours at 37°C. 3 ml of cold Gel-PBS was added to each tube, cells were centrifuged, and the media was measured for LH content. The four pretreatment groups were evaluated for their ability to synthesize and secrete LH immediately after treatment and after the 24 hour recovery period. The results of one experiment are shown in Table III.

TABLE III

LH Synthesis and Release by Ovine Pituitary Cells
(ng per 5 x 10^{6} cells)

TREATMENT[1]	SYNTHESIS[2]	INDUCED RELEASE[3]
CONTROL	526.3	723.9
10^{-8} M GnRH	545.5	320.3
PAP	137	26.2
PAP-D-Lys^{6}	0	11.7

[1] Cells were incubated with the various treatments for 16 hours.

[2] Synthesis of LH was measured during a 24 hour period of culture after the agents were removed from the cells.

[3] Release of LH was induced by maximal dose of GnRH (10^{-7} M).

These data, although obtained with the least promising of our conjugates, reveal a large and specific effect of PAP-SPDP-[D-Lys^{6}, desGly^{10}]-GnRH-ethylamide (ethylamide is abbreviated as "EA" in Table III) on the gonadotropes ability to synthesize and secrete LH. It is not possible to determine whether the gonadotropes were specifically killed as they comprise <10% of the total number of pituitary cells, but the data strongly suggest the conjugate disrupted their normal function.

Applicants then tested the more promising Barley-2IT-[D-Lys^{6}, desGly^{10}]-GnRH-ethylamide conjugate in similar assay systems. Table IV shows the results of a similar experiment. Ovine pituitary cells were again placed in culture with various agents and the

total LH in the cells and media determin d after a 24 hour exposure, wash, and further 24 hour culture in standard media.

TABLE IV

Total Culture LH after Exposure to GnRH and Toxin Conjugates with or without Lysosomal Agents

Incubation Condition	Total LH(Ng/10^5 cells in Culture)
Control	1.90
D-Lys^{6}GnRH-EA	1.62
Barley Toxin	1.49
Barley Toxin-2IT-D-Lys^{6}GnRH-EA	.91
Barley Toxin-2IT-D-Lys^{6}GnRH-EA + Monensin	1.83
Barley Toxin-2IT-D-Lys^{6}GnRH-EA + Chloroquine	.62
Barley Toxin-2IT-D-Lys^{6}GnRH-EA + NH_4Cl	1.33
Barley Toxin-2IT-D-Lys^{6}GnRH-EA + Killed Adenovirus	1.13

These results indicate a specific killing effect of the toxin conjugate after only 24 hours of exposure. The lysosomally active agents do not potentiate this effect with the exception of chloroquine. When such experiments are combined with secondary challenge by GnRH, it appears that few gonadotropes are able to synthesize new GnRH after exposure to the barley toxin conjugate (data not shown).

7. In vivo Experiments. Several experiments were done to determine the effects of the pokeweed toxin (PAP)-SPDP-[D-Lys^6, desGly^{10}]-GnRH-ethylamide conjugate in adult Sprague Dawley rats. Groups of 5-7 rats were treated with 20 ng of analogue; 20 ng conjugate (receptor binding assay equivalents), saline, or a conjugate made from a protein of similar molecular weight to the pokeweed toxin (carbonic anhydrase or ovalbumin). The most effective time course was found to be weekly injections for 4 weeks. The effect of

such treatment was monitored in several ways. The ability of th animals to respond to a GnRH analogue challenge by measuring LH and/or serum testosterone levels 30-90 minutes after injection was followed. No difference was found among the groups. This result might be expected, since inducible LH release in intact animals is quite small secondary to chronic feedback suppression by the testicular androgens. Secondly, applicants followed gonad weights and found the testes in the PAP-[D-Lys^6, desGly^{10}]-GnRH-ethylamide group to be decreased by 50%, although the control conjugates had similar effects. The PAP and carbonic anhydrase conjugate groups were found to be infertile in breeding tests, indicating a potential effect of this enzyme on testis tissue. Interestingly, light microscopy of these animals revealed no changes in the pituitaries, but interstitial (Leydig) cell depletion in the PAP-SPDP-[D-Lys^6, desGly^{10}]-GnRH-ethylamide treated group, indicating a possible specific cellular effect on rat testicular function. This was not surprising since there are GnRH receptors on Leydig cells in the rat testis.

Applicants also tested the PAP-carbodiimide-[D-Lys^6, desGly^{10}]-GnRH-ethylamide conjugate in ovariectomized female rats. In contrast to the SPDP conjugate, and in this system where gonadal feedback is not a problem, this drug appears capable of producing a 15 fold decrease in the serum LH response to GnRH analogue challenge (Figure 1A or 1B), again indicating the importance of applicant's studies on various linking techniques.

Figure 1B indicates the results of a challenge by one of applicants' compounds to ovariectomized rats. Serum concentrations of LH in ovariectomized rats

treated with saline (hatched bars) or pokeweed anti-viral protein conjugated to a GnRH super-agonist (solid bars) are d picted. Th open space above the bars indicates the amount of LH released in response to a GnRH challenge. The challenges were administered on the first day of treatment and again 4 weeks later. Compared to control there was greater than a 90% reduction in LH release after GnRH challenge at 4 weeks of treatment.

Based on the above data (with regards to LH synthesis inhibition) applicants then carried out experiments in intact male and female rats. Animals received 4 injections at 3 day intervals of PAP-CI-[D-Lys^{6}, desGly^{10}]-GnRH-ethylamide or of the GnRH analogue or toxin alone or saline. Conjugate treated male animals (but not control) showed a 50% reduction in fertility (i.e., 50% of females exposed to these male animals became pregnant, compared to 100% of controls). Histologic examination of the testes of experimental animals revealed residual spermatogenesis in about 10% of tubules. In conjugate treated female animals, fertility was abrogated for more than 4 months following treatment. There were no side effects noted from these injections.

To further understand the effect of hemitoxins and conjugates on non-target tissues, applicants initiated studies on the tissue distribution of ^{125}I-toxin-conjugates and have demonstrated important differences among the toxins in (for example) concentration in the kidneys, indicating the importance of testing the various proteins to avoid potential non-target tissue toxicity. For example, applicants have found that the tissue/serum ratio of unconjugated PAP 2 hours after injection for various organs ranges from .03 in brain to 85.5 in kidney. In contrast, unconjugated barley

hemotoxin is 8-fold less concentrated in kidney (see Table IV). Conjugation with the GnRH analogue alters these ratios consid rably.

TABLE V

Tissue Distribution of Hemitoxins and Hemotoxin Conjugates

Tissue	PAP	PAP-SPDP-D-Lys^{6}GnRH
Pituitary	.20	.11
Brain	.03	.01
Adrenal	.48	.02
Kidney	85.5	12.6
Liver	2.48	1.07
Spleen	2.29	.73
Testis	.03	.02

Tissue/Serum Ratio of Labeled Protein

GnRH	Barley	Barley-CI-D-Lys^{6}GnRH
Pituitary	1.08	1.06
Brain	.04	.04
Adrenal	.70	1:5
Kidney	10.5	4.0
Liver	.43	3.52
Spleen	.4	5.07
Testis	.10	.10

Thus these experiments produced a group of compounds capable of sterilizing (temporarily or permanently) animals by destroying the gonadotrophs of an animal's anterior pituitary gland. These compounds can be administered in the form of pharmaceutically acceptable, and otherwise nontoxic salts. It should also be noted that these compounds can be administered individually, or in combination with each other, to animals intravenously, subcutaneously, intramuscularly or orally to achieve fertility inhibition and/or control. Preferably administration will be intravenous or intramuscular in a suitable carrier

such as, for exampl , in isotonic saline phosphate buffer s lutions or the like. They also can be used in applications calling for reversible suppression of gonadal activity, such as for the management of precocious puberty or during radiation or chemotherapy. Effective dosages will vary with the form of administration and the particular species of mammal being treated. An example of one typical dosage form is a physiological saline solution containing the peptide which solution is administered to provide a dose in the range of about 0.1 to 10 mg/kg of body weight.

Although the invention has been described with regard to its preferred embodiments, it will be apparent to those skilled in this art, upon reading the above detailed description and examples, that various modifications and extensions can be made thereto without departing from the spirit of the present invention and that the scope of said invention shall be limited only by the scope of the appended claims.

Thus having disclosed our invention, we claim:

1. A method for sterilizing an animal, said method comprising administering an effective amount of a compound comprised of a peptide hormone group capable of binding with a receptor cell of the animal's pituitary gland and wherein said peptide hormone is selected from the group consisting of a GnRH analog (GnRH-A), human chorionic gonadotropin, equine chorionic gonadotropin, luteinizing hormone and follicle - stimulating hormone, which peptide hormone is conjugated to a toxin group capable of destroying said receptor cell of the pituitary gland when said toxin group is conjugated to said peptide hormone.

2. The method of claim 1 wherein the animal is selected from the group of animals consisting of mammals, fowl and fish.

3. A method for sterilizing an animal, said method comprising administering an effective amount of a compound comprised of a peptide hormone group capable of binding with a receptor cell of the animal's pituitary gland and wherein said peptide hormone is selected from the group consisting of a GnRH analog (GnRH-A), human chorionic gonadotropin, equine chorionic gonadotropin, luteinizing hormone and follicle - stimulating hormone, which peptide hormone is conjugated to a toxin group capable of destroying said receptor cell of the pituitary gland and wherein said toxin group is selected from the group consisting of the plant toxins: ricin, modeccin, abrin, pokeweed anti-viral protein, α-amanitin, gelonin ribosome inhibiting protein ("RIP") barley RIP, wheat RIP, corn RIP, rye RIP and flax RIP; the bacterial toxins: diptheria toxin, pseudomonas exotoxin and shiga toxin

and the chemical toxins: m lphalan, methotrexate, nitrogen mustard, doxorubicin and daunomycin.

4. The method of claim 3 wherein the animal is selected from the group of animals consisting of mammals, fowl and fish.

5. A method for sterilizing an animal, said method comprising administering an effective amount of a compound having the formula:

```
pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Z
                      |
                      X
                      |
                      Y
                      |
                      T
```

wherein X is an amino acid selected from the group consisting of tyrosine, D-tyrosine, Ornithine, D-ornithine, glutamic acid, D-glutamic acid, aspartic acid, D-aspartic acid, cysteine and D-cysteine; Y is a linking agent selected from the group consisting of: carbodiimide, 2-iminothiolane, SPDP, (N-succinimidyl 3-(2-pyridyldithio) propionate), bis-diazobenzidine and glutaraldehyde; Z is a chemical group selected from the group consisting of Gly-NH_2, ethylamide, and Aza-Gly-NH_2 and T is a toxin group selected from the group consisting of the plant toxins: ricin, modeccin, abrin, pokeweed anti-viral protein, α-amanitin, gelonin ribosome inhibiting protein ("RIP") barley RIP, wheat RIP, corn RIP, rye RIP and flax RIP; the bacterial toxins: diptheria toxin, pseudomonas exotoxin and shiga toxin and the chemical toxins: melphalan, methotrexate, nitrogen mustard, doxorubicin and daunomycin.

6. A method for treating sex horm ne r lated diseas s of a mammal, said method comprising administering an effective amount of a compound comprised of a peptide hormone group capable of binding with a receptor cell of the mammal's pituitary gland and wherein said peptide hormone is selected from the group consisting of a GnRH analog (GnRH-A), human chorionic gonadotropin, equine chorionic gonadotropin, luteinizing hormone and follicle - stimulating hormone, which peptide hormone is conjugated to a toxin group capable of destroying said receptor cell of the pituitary gland when said toxin group is conjugated to said peptide hormone.

7. A method for treating sex hormone related diseases of a mammal, said method comprising administering an effective amount of a compound comprised of a peptide hormone group capable of binding with a receptor cell of the mammal's pituitary gland and wherein said peptide hormone is selected from the group consisting of a GnRH analog (GnRH-A), human chorionic gonadotropin, equine chorionic gonadotropin, luteinizing hormone and follicle - stimulating hormone, which peptide hormone is conjugated to a toxin group capable of destroying said receptor cell of the pituitary gland and wherein said toxin group is selected from the group consisting of the plant toxins: ricin, modeccin, abrin, pokeweed anti-viral protein, α-amanitin, gelonin ribosome inhibiting protein ("RIP") barley RIP, wheat RIP, corn RIP, rye RIP and flax RIP; the bacterial toxins: diptheria toxin, pseudomonas exotoxin and shiga toxin and the chemical toxins: melphalan, methotrexate, nitrogen mustard, doxorubicin and daunomycin.

8. A method for treating sex hormone related diseases of a mammal, said method comprising administering an effective amount of a compound having the formula:

$$\begin{array}{c} \text{pGlu-His-Trp-Ser-Tyr-}\overset{}{\text{Gly}}\text{-Leu-Arg-Pro-Gly-Z} \\ \quad | \\ \quad \text{X} \\ \quad | \\ \quad \text{Y} \\ \quad | \\ \quad \text{T} \end{array}$$

wherein X is an amino acid selected from the group consisting of tyrosine, D-tyrosine, ornithine, D-ornithine, glutamic acid, D-glutamic acid, aspartic acid, D-aspartic acid, cysteine and D-cysteine; Y is a linking agent selected from the group consisting of: carbodiimide, 2-iminothiolane, SPDP, (N-succinimidyl 3-(2-pyridyldithio) propionate), bis-diazobenzidine and glutaraldehyde; Z is a chemical group selected from the group consisting of Gly-NH_2, ethylamide, and Aza-Gly-NH_2 and T is a toxin group selected from the group consisting of the plant toxins: ricin, modeccin, abrin, pokeweed anti-viral protein, α-amanitin, gelonin ribosome inhibiting protein ("RIP") barley RIP, wheat RIP, corn RIP, rye RIP and flax RIP; the bacterial toxins: diptheria toxin, pseudomonas exotoxin and shiga toxin and the chemical toxins: melphalan, methotrexate, nitrogen mustard, doxorubicin and daunomycin.

9. A method for increasing the rate of protein formation on an animal raised for its meat, said method comprising administering an effective amount of a compound having the formula:

```
pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-Z
                      |
                      X
                      |
                      Y
                      |
                      T
```

wherein X is an amino acid selected from the group consisting of tyrosine, D-tyrosine, ornithine, D-ornithine, glutamic acid, D-glutamic acid, aspartic acid, D-aspartic acid, cysteine and D-cysteine; Y is a linking agent selected from the group consisting of: carbodiimide, 2-iminothiolane, SPDP, (N-succinimidyl 3-(2-pyridyldithio) propionate), bis-diazobenzidine and glutaraldehyde; Z is a chemical group selected from the group consisting of Gly-NH_2, ethylamide, and Aza-Gly-NH_2 and T is a toxin group selected from the group consisting of the plant toxins: ricin, modeccin, abrin, pokeweed anti-viral protein, α-amanitin, gelonin ribosome inhibiting protein ("RIP") barley RIP, wheat RIP, corn RIP, rye RIP and flax RIP; the bacterial toxins: diptheria toxin, pseudomonas exotoxin and shiga toxin and the chemical toxins: melphalan, methotrexate, nitrogen mustard, doxorubicin and daunomycin.

10. The method of claim 9 wherein the animal is selected from the group of animals consisting of mammals, fowl and fish.

11. A compound, used for destroying gonadotrophs of the pituitary gland, said compound having the formula:

$$\begin{array}{c} \text{pGlu-His-Trp-Ser-Tyr-}\underset{\begin{array}{c}|\\ \text{X}\\ |\\ \text{Y}\\ |\\ \text{T}\end{array}}{\text{Gly}}\text{-Leu-Arg-Pro-Gly-Z} \end{array}$$

wherein X is an amino acid selected from the group consisting of tyrosine, D-tyrosine, ornithine, D-ornithine, glutamic acid, D-glutamic acid, aspartic acid, D-aspartic acid, cysteine and D-cysteine; Y is a linking agent selected from the group consisting of: carbodiimide, 2-iminothiolane, SPDP (N-succinimidyl 3-(2-pyridyldithio) propionate), bis-diazobenzidine and glutaraldehyde; Z is a chemical group selected from the group consisting of Gly-NH_2, ethylamide, and Aza-Gly-NH_2 and T is a toxin group selected from the group consisting of the plant toxins: ricin, modeccin, abrin, pokeweed anti-viral protein, α-amanitin, gelonin ribosome inhibiting protein ("RIP") barley RIP, wheat RIP, corn RIP, rye RIP and flax RIP; the bacterial toxins: diptheria toxin, pseudomonas exotoxin and shiga toxin and the chemical toxins: melphalan, methotrexate, nitrogen mustard, doxorubicin and daunomycin.

12. The compound of claim 11 where the toxin group T is attached to the linkage group Y at a point on the Y group which comprises a chemical group selected from the group consisting of an amino group, a carboxylic group or a sulfhydryl group.

13. The compound of claim 11 wherein the linkage group Y is attached to the amino acid group X at a point on the amino acid group X which comprises a chemical group selected from the group consisting of

an amino group, a carboxylic group or a sulfhydryl group.

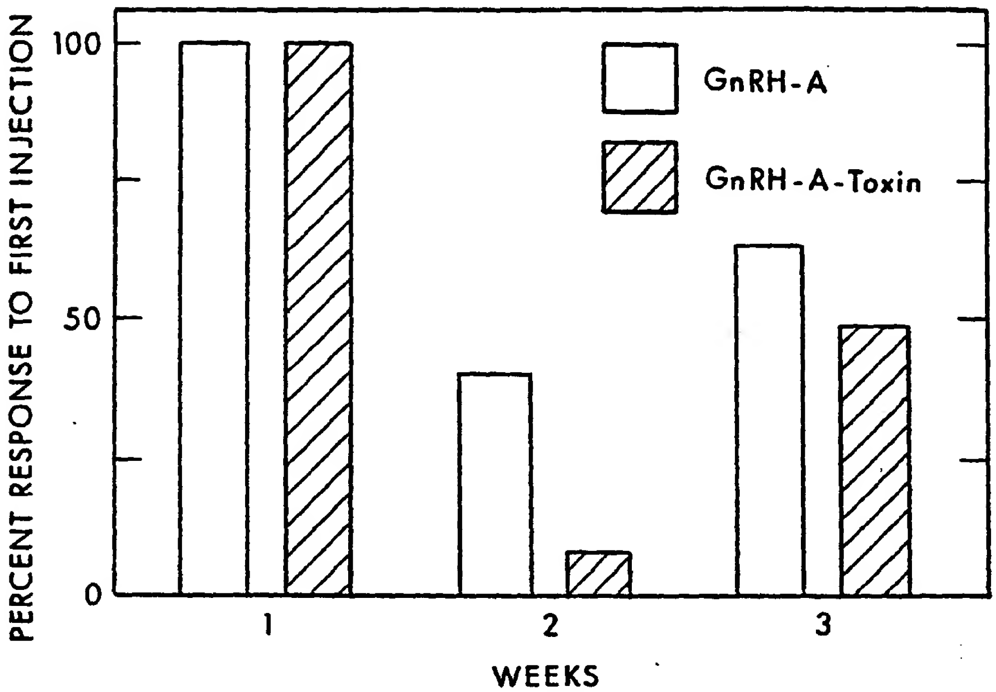
14. A compound used for destroying gonadotrophs of the pituitary gland, wherein said compound is comprised of a hormone selected from the group consisting of (pyro)Glu-His-Trp-Ser-Tyr-D-Lys-Leu-Arg-Pro-Ethylamide D-Lys^5-GnRH-Pro^9-Ethylamide, a luteinizing hormone or an interstital-cell stimulating hormone, a linking agent selected from the group consisting of succinimidyl 4-(p-maleimidophenyl) butyrate (SPDP) and N-Succinimidyl 3-(2-pyridyldithio) proprionate (SMPB) and a toxin selected from the group consisting of diphtheria toxin A chain and pseudomonas exotoxin A chain.

15. A compound used for destroying gonadotrophs of the pituitary gland, said compound being a D-Lys^6-GnRH-SMPB-diphtheria toxin A chain conjugate.

16. A compound used for destroying gonadotrophs of the pituitary gland, said compound being a D-Lys^6-GnRH-SMPB-pseudomonas exotoxin A chain conjugate.

Fig. 1

A


PERCENT RESPONSE TO FIRST INJECTION
100
50
0
GnRH-A
GnRH-A-Toxin
1
2
3
WEEKS

B

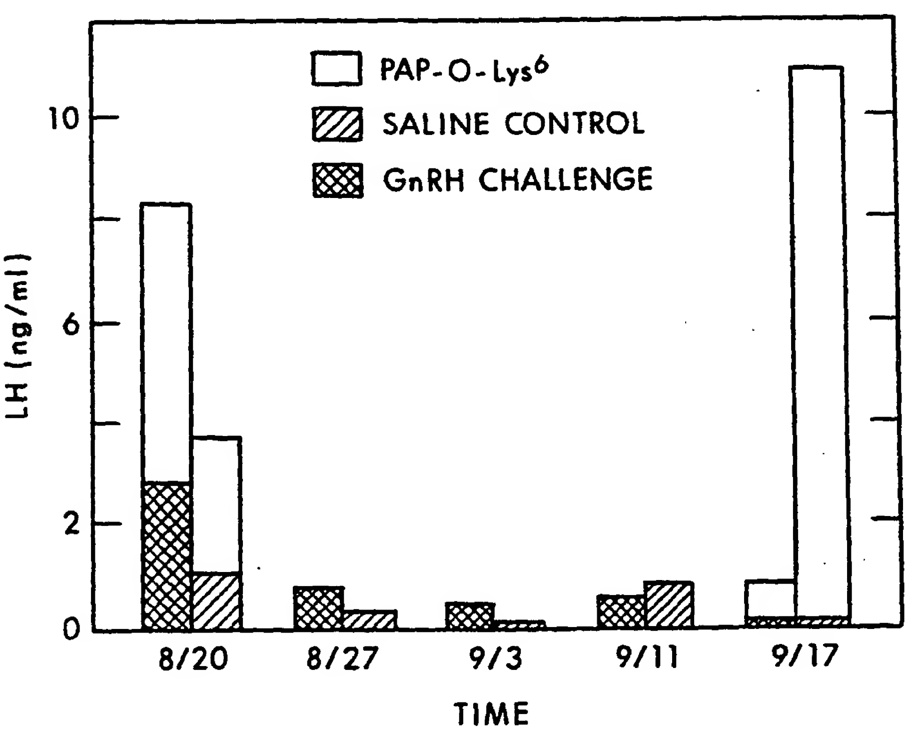

LH (ng/ml)
10
6
2
0
PAP-O-Lys⁶
SALINE CONTROL
GnRH CHALLENGE
8/20
8/27
9/3
9/11
9/17
TIME

Fig. 2

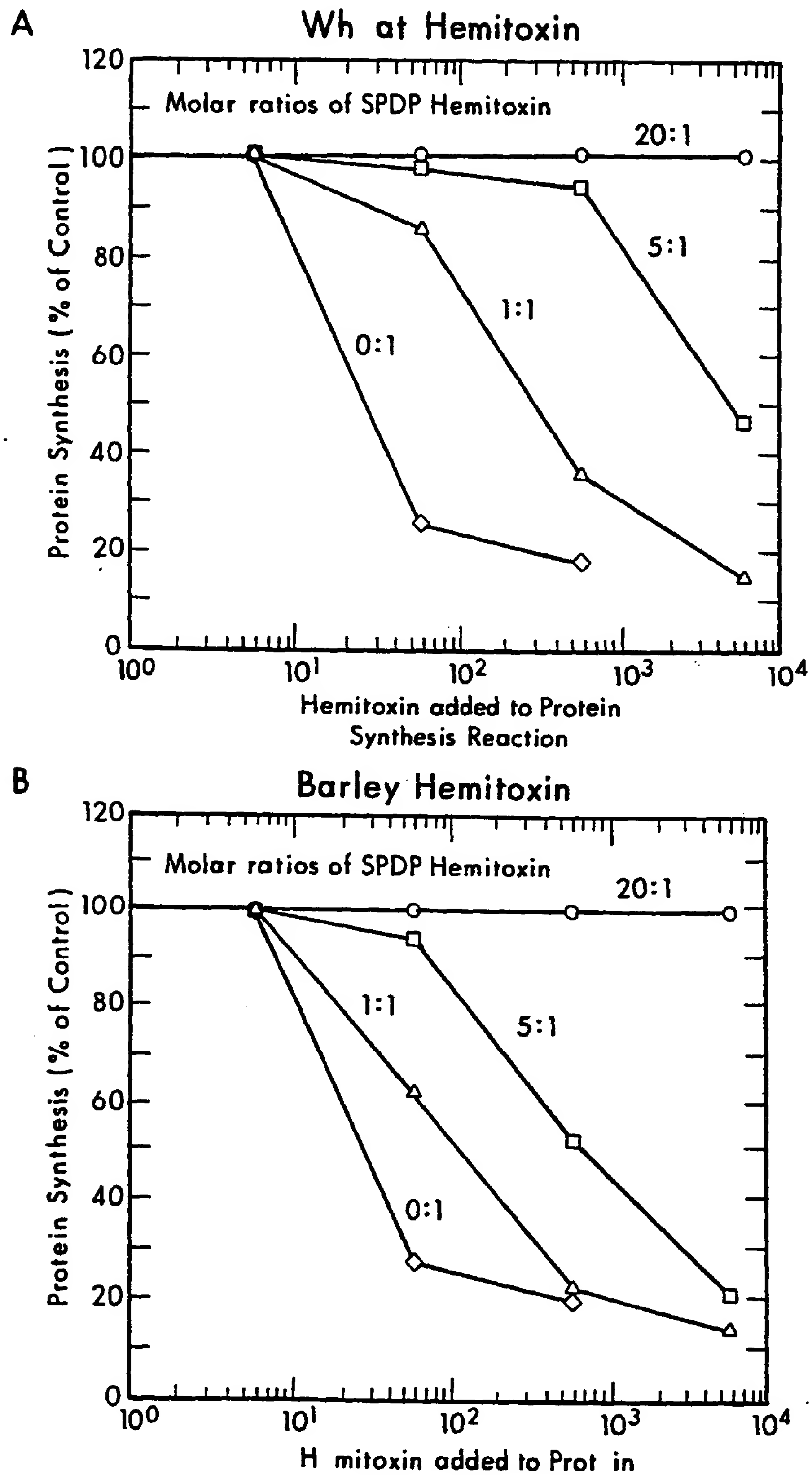

A
Wh at Hemitoxin
Molar ratios of SPDP Hemitoxin
20:1
5:1
1:1
0:1
Protein Synthesis (% of Control)
120
100
80
60
40
20
0
10^0
10^1
10^2
10^3
10^4
Hemitoxin added to Protein Synthesis Reaction
B
Barley Hemitoxin
Molar ratios of SPDP Hemitoxin
20:1
1:1
5:1
0:1
Protein Synthesis (% of Control)
120
100
80
60
40
20
0
10^0
10^1
10^2
10^3
10^4
H mitoxin added to Prot in

Fig. 3

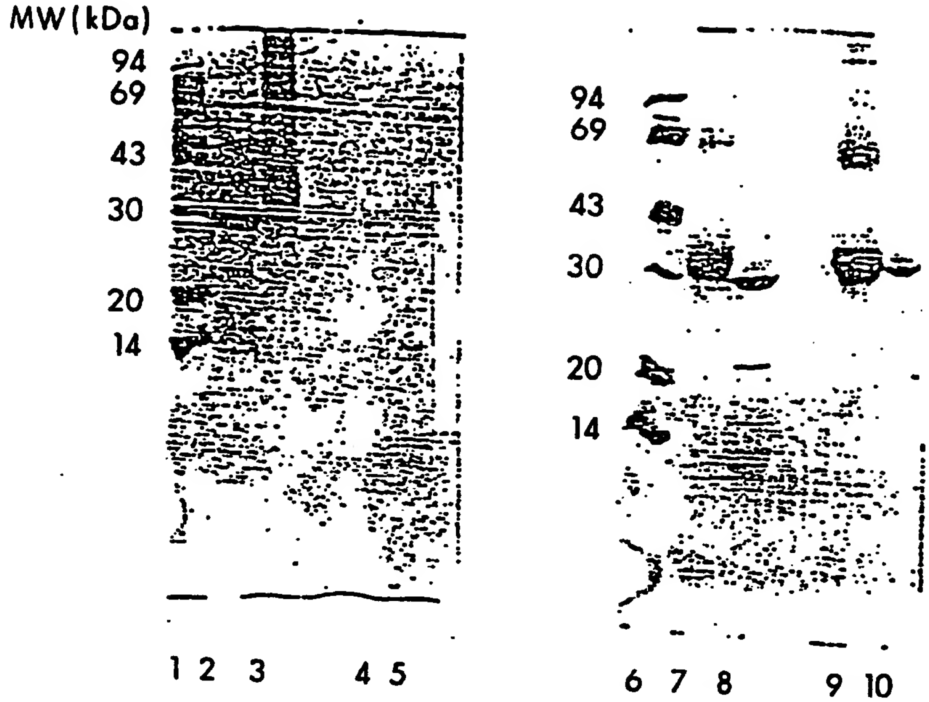


Fig. 4

Effect of 2-Iminothiolane Conjugation on Barley Toxin Activity

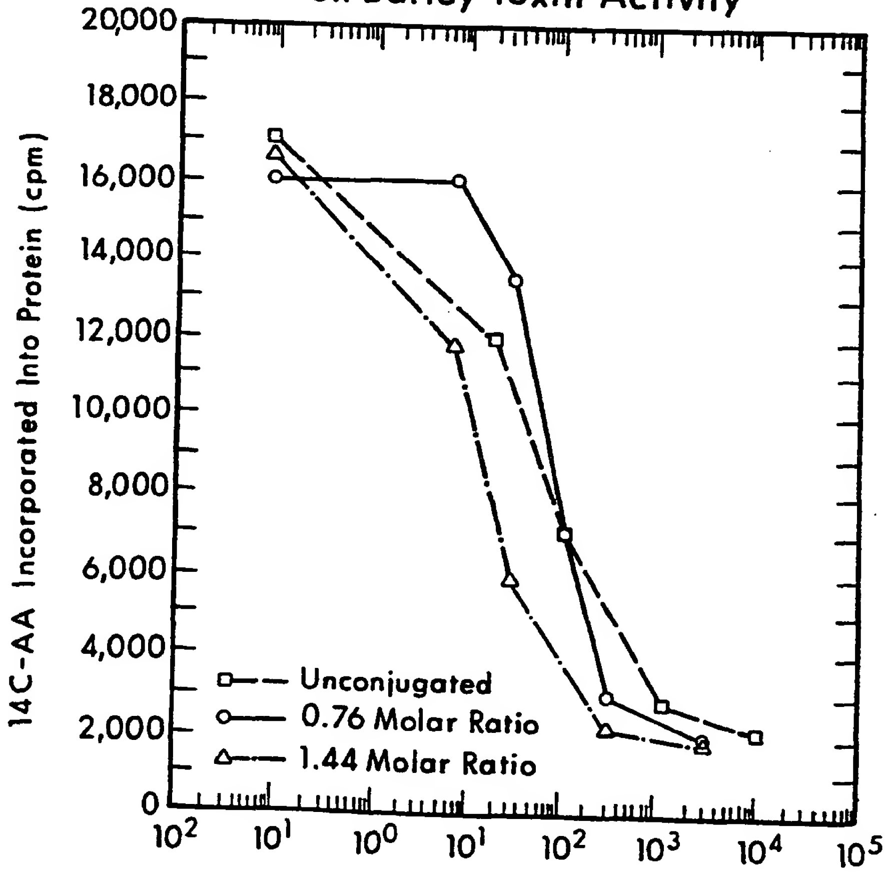


Fig. 4a

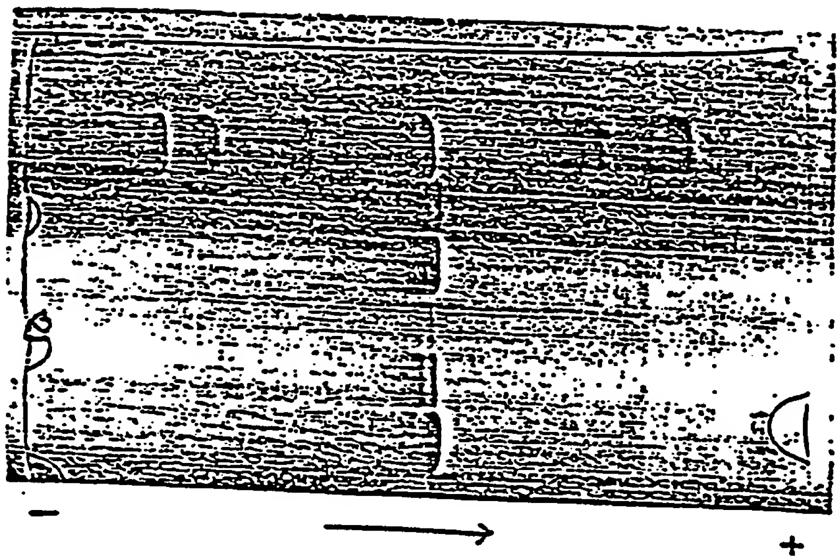
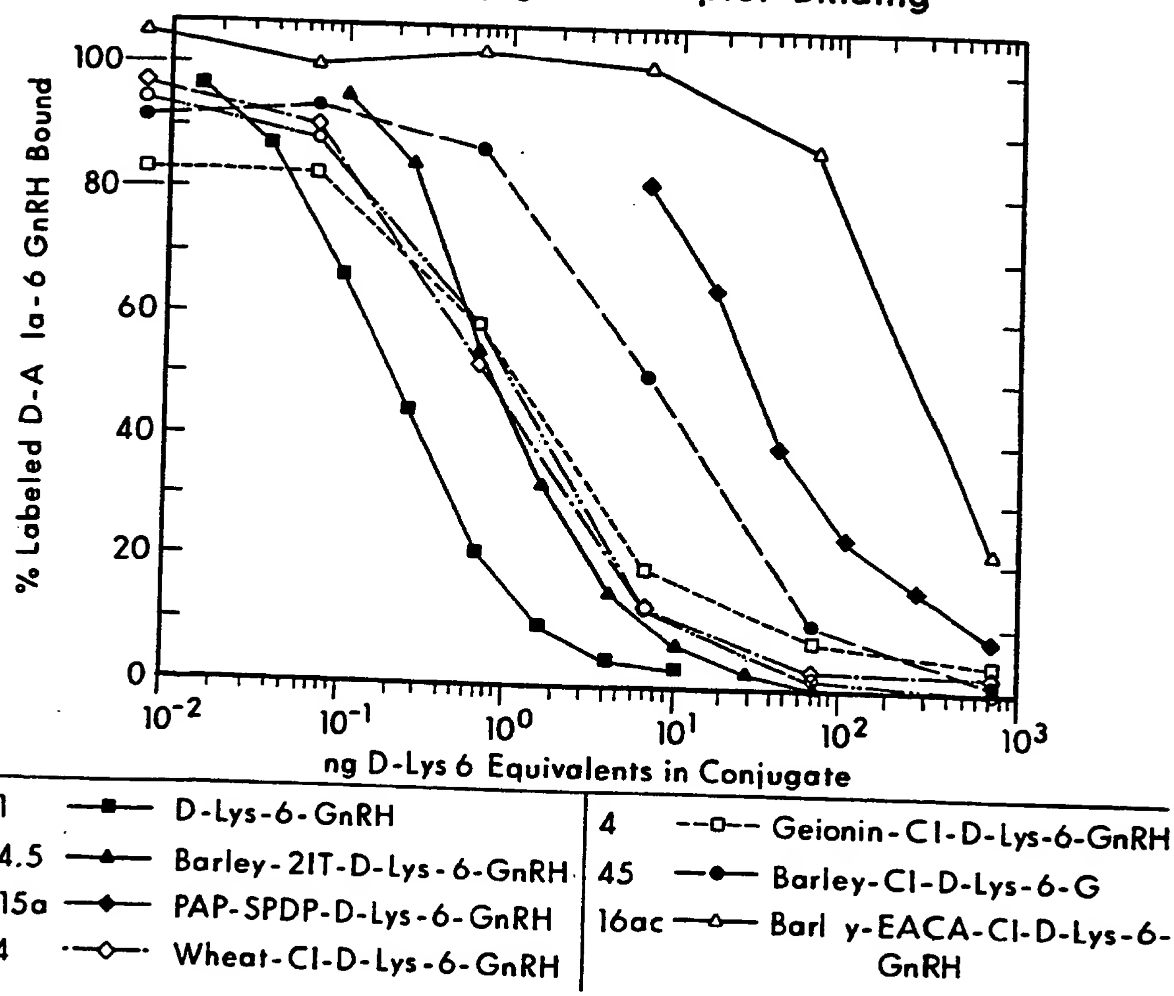


Fig. 5

Toxin Conjugate Receptor Binding



INTERNATIONAL SEARCH REPORT

International Application No. PCT/US90/01038

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (5) A61K 37/02, 43: C07K 7/06, 08; C07K 17/06

U.S. 514/ 12, 13, 14, 15; 530/324, 325, 326, 327, 409

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S.	514/12, 13, 14, 15 530/324, 325, 326, 327, 409

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
A	US, A 4,081,533 (Cheesman) 28 MARCH 1978. See abstract.	1-16
A	US, A, 4,201,770 (Stevens) 06 MAY 1980. See abstract.	1-16
A	US, A, 4,302,386 (Stevens) 24 NOVEMBER 1981. See abstract.	1-16
A	US, A, 4,526,716 (Stevens) 02 July 1985. See abstract.	1-16
X Y	US, A 4,691,006 (Stevens) 01 September 1987. See abstract and claims.	1-4,6-7 5,8,9-16
X Y	US, A, 4,767,842 (Stevens) 30 August 1988. See abstract adn claims.	1-4,6-7 5,8,9-16

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
17 MAY 1990	20 JUL 1990
International Searching Authority	**Signature of Authorized Officer**
ISA/US	LESTER L. LEE

Form PCT/ISA/210 (second sheet) (Rev.11-87)